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13. ABSTRACT ***(Maximum 200 Words)***

The development and progression of cancer result from multiple genetic changes accumulated in the cells. The identification of tumor suppressor genes inactivated and proto-oncogenes activated in mammary epithelial cells is essential to understand the genetic basis of breast cancer and is a prerequisite for development of strategies for prevention, diagnosis, and treatment. In breast cancer, loss of heterozygosity (LOH) was detected frequently on chromosome 17 and other chromosomes, suggesting unrecognized tumor suppressor genes. We are applying the novel retroviral-tagging strategy to identify the genes using chromosome 17-suppressed (independent of p53 and BRCA1) breast cancer cell lines. In contrast to the parental tumorigenic cell line CAL51, the suppressed sublines CAL/17-1, CAL/17-3 and CAL/17-5 display insulin-dependent growth in flasks, no growth in soft-agar culture and athymic nude mice. In this annual report, we present our results on selection for the anchorage-independent cell sublines induced by retroviral transduction of the chromosome 17-mediated suppressed cell lines CAL/17, in addition to successful selection for insulin-independent cell sublines. We are now in the process to conduct tumorigenicity tests in athymic nude mice and to clone genomic sequences flanking the integrated retroviral vectors.

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(5) INTRODUCTION

Breast cancer is the most common malignancy in Western women, affecting up to one in 10 women during their lifetime and approximately 40,000 women dying from the disease each year in the U.S. The development of breast cancer is believed to result from multiple genetic changes accumulating in mammary epithelial cells. In searching for the genetic changes, increasing interest has been focused on tumor suppressor genes involved in protection (or control) of normal mammary epithelial cells from tumorigenic transformation.

While many investigators have observed LOH in breast cancers on chromosomes 1p, 1q, 3p, 6q, 7q, 11p, 13q, 16q, 17p, 17q, and 18q at the high frequencies variable from 20-60%, chromosome 17 is one of the most frequent carriers of LOH (1-19). On 17p, two distinct regions, 17p13.1 (containing *p53*) and 17p13.3, have shown LOH with frequencies ranging from 30-60% and 60-70%, respectively (10-11, 20-26). On 17q, three regions of frequent LOH have been identified. 17q21 LOH (13, 26-30) contains *BRCA1* (31). LOH at 17q11.1-q12 was detected as frequently as 79% in sporadic breast cancer (32). The third LOH is telomeric to *BRCA1* (26, 29). These studies demonstrate that, in addition to *p53*, *BRCA1, BRCA2*, and others, chromosome 17 and other chromosomes with the high frequent areas of LOH harbor unrecognized tumor suppressor genes involved in the control of the normal growth of mammary epithelial cells.

Direct evidence supporting the existence of additional breast cancer suppressor genes comes from introduction of a *neo*-tagged chromosome 17 into breast cancer cell lines by microcell-mediated chromosome transfer that demonstrated suppression of tumorigenicity (33-37). Casey *et al.* was the first to provide biological evidence that *in vitro* growth of the breast cancer cell line MCF 7 (carrying wild-type *p53*) was suppressed by the introduction of a *neo*-tagged chromosome 17 (33). A very similar result was independently reported by Negrini *et al.* (34). In addition, anchorage-independent growth, cell growth rate on plastic plates, and tumorigenicity in athymic nude mice of the mammary carcinoma cell line R30 were suppressed by introduction of chromosome 17. Wild-type *p53* was not involved in this suppression (35). Furthermore, only the long arm of the transferred chromosome 17 was capable of suppressing the tumorigenicity of the *p53*-mutant breast cancer cell line MDA-MD-231 (34). Finally, Theile *et al.* demonstrated that suppression of tumorigenicity of the breast cancer cell line CAL51 by an introduced chromosome 17 did not require transfer of *p53* or *BRCA1* (37). Thus, additional tumor suppressor genes on chromosome 17 have yet to be identified.

To facilitate identification of tumor suppressor genes we have developed a novel strategy to reverse monochromosome-mediated tumor suppression by retroviral insertional mutagenesis and/or functional inactivation mediated by expressed cDNA fragments (38). Insertional mutagenesis disrupts tumor suppressor genes (e.g., *APC* for familial adenomatous polyposis [39] and *p53* in osteosarcoma [40]) and has been used as a powerful tool to identify various genes including *Fli-1*, *p53*, *erb-B*, and *myc* (41-43), *vin-1*/*cyclin D2* (44), *Tiam-1* (45), *bcar-1,* and *CRL-1* (46-47). Furthermore, it is known that retroviral insertion could activate proto-oncogene. Finally, functional inactivation mediated by expressed cDNA fragments has been achieved by knockout of gene function (48) and by methods designed to identify genetic suppressor elements by antisense cDNA

or dominant negative mutant proteins (49-51). Combining all components of these established approaches into a single system of retroviral-vector-cDNA, we have developed a novel strategy for identification of genetic loci and tumor suppressor genes (38).

"Suppression and reversion of suppression" are the two basic aspects of this novel strategy. "Suppression" refers to the suppression of tumorigenic phenotypic features including anchorage-independent growth, focus formation in plastic culture, rapid cell population doubling time, and tumor formation in athymic nude mice by introduction of a *neo*-tagged monochromosome into a cancer cell line via a microcell mediated chromosome transfer. "Reversion of suppression" means the reversion to the tumorigenic phenotype induced by insertional mutagenesis (proviral tagging) and/or functional inactivation of the suppressor gene(s) by antisense or dominant negative mutant proteins following the transduction of a retrovirus expression vector-carried cDNA library into the monochromosome suppressed cells.

The hypothesis underlying this approach is the following. (1) The phenotypic reversion can derive from inactivation of tumor suppressor genes. The suppression related genetic locus or loci on the introduced chromosome provide targets to insertional mutagenesis. In addition, derived from cancer cell lines, the suppressed sublines may carry many mutated genetic loci that leave functional counterparts being "haploid" targets to insertional mutagenesis. Furthermore, the suppressor gene products can be targets to functional inactivation by antisense and mutant proteins. The poly(A)$^+$-RNA from the suppressed cancer cell line is used to construct cDNA library and then re-introduced to the suppressed cell line to induce the reversion, reasoning that cDNA fragments of the suppressor genes would be present in the library providing antisense and mutant proteins to inhibit activities of the suppressor genes. It is well known that the phenotypic reversion can also come from activation of proto-oncogenes. (2) The successfully transduced tumorigenic cells can be positively selected in soft agar culture following co-selection for the drug-resistance genes on both the suppressive chromosome and the retroviral vector. (3) The cDNA and genomic sequences tagged to the vectors and involved in the tumorigenic reversion can be readily isolated by PCR-based techniques. Based on this hypothesis we have successfully generated a serial retroviral-tagged revertant cell sublines using the chromosome-6 suppressed melanoma cell line UACC903(+6) (38).

We are applying the same strategy to identify unrecognized tumor suppressor gene(s) from a chromosome-17 suppressed breast cancer cell sublines. This study is using the tumorigenic cell line CAL51 and the chromosome-17 suppressed cell sublines CAL/17-1, CAL/17-3, and CAL17-5 (37). The parental CAL51 cell line demonstrates insulin-independent growth, anchorage-independent growth, and rapid formation of subcutaneous tumors in athymic nude mice. All these readily detectable phenotypic features are suppressed in the chromosome 17 containing cell sublines CAL/17-1, CAL17-3 and CAL17-5 (37). The central goal of this project is to identify breast cancer suppressor genes. The specific aims include (1) use of the chromosome-17 suppressed breast cancer cell sublines CAL/17-1, CAL17-3 and CAL17-5 (37) to generate the anchorage-independent revertants by transduction of a retroviral expression vector-carried cDNA library and (2) use of the anchorage-independent revertants to identify previously unrecognized suppressor genes in breast cancer. We now report our results on the selection

for anchorage-independent cell sublines, in addition to successful selection for the insulin-independent cell sublines.

(6) BODY

Below are the timetable and the technique objectives of the **first-two years** from our original proposal. The current status of the technique objectives is indicated in the parentheses. The detailed description follows the Statement of Work.

Table 1. The Timetable for the Proposed Experiments

Specific Aim 1	Specific Aim 2
Year 01	
- Completion of the library construction	
- Completion of the retrovirus package	
- Completion of the retrovirus transduction	
Year 02	
- Completion of soft agar selection for colony forming cells and establishment of revertant sublines	
- Study of *in vitro* growth and tumorigenicity test	
- Identification of the effective cDNAs	

Technique Objectives

Task 1: Months 1-2: Cell culture of the CAL51 and CAL/17-1 cells. Isolation of poly(A)$^+$RNA from the chromosome-17 suppressed cell line CAL/17-1 for construction of a cDNA library. Isolation of DNA and total RNA from the two cell sublines for the future Southern and Northern analysis. **(Completed)**.

Task 2: Months 2-3: Transfection of pLM2 plasmid vector into CAL51 and CAL/17-1 cells to determine the killing curves in the presence of L-histidinol dihydrochloride. **(Completed)**.

Task 3: Months 3-4: Soft agar culture of the L-histidinol-resistant CAL51 and CAL/17-1 sublines (transfected with pLM2 vector) to generate the first-hand data for soft agar selection experiments. **(Completed)**.

Task 4: Months 3-5: Construction of a random primed normalized cDNA library onto pLM2 vectors using poly(A)$^+$RNA isolated from CAL/17-1 cells. **(Modified and completed)**.

We have constructed the randomly-primed cDNA library as reported in the previous fiscal year.

Task 5: Months 6-8: Package of the ecotropic and amphotropic retrovirus particles from the pLM2-carried cDNA library and determination of a titer of the retrovirus particles. **(Completed)**.

Task 6: Months 9-11: Transduction of the retrovirus particles into the CAL/17-1 cells and selection for colony forming cells using soft agar culture. **(Completed).**

Task 7: Months 11-14: Individual colonies will be lifted from soft agar culture. Cells from the individual colonies will be expanded on plastic culture and re-plated in soft agar culture to eliminate false positives and to establish true revertant sublines. **(Nearly completed).**

Colonies have been lifted from soft agar culture. The cells from the colonies are in expansion for re-plating in soft agar culture to eliminate false positives and to establish true revertant sublines.

Task 8: Months 14-16: Study of *in vitro* growth of anchorage-independent revertant cell sublines to select candidates for test of tumorigenicity in athymic nude mice. **(To be done).**

Task 9: Months 17-20: Tumorigenicity tests of the candidate sublines in athymic nude mice to identify the tumorigenic sublines. **(To be done).**

Task 10: Months 19-23: Identification of cDNA inserts on the integrated retroviral vectors. Test of effects of identified cDNAs on tumorigenic reversion of the CAL/17-1 cell line. **(To be done).**

Task 11: Months 21-24: Cloning of genomic sequences flanking the integrated retrovirus vectors and subsequent identification of their encoded or adjacent cDNAs. **(To be done).**

In addition, after the retroviral transduction, we have successfully selected for insulin-independent cell sublines from the chromosome 17-mediated suppressed cell line CAL/17-1. Since the seven insulin-independent cell sublines display the significantly different growth rates, it is likely that the retroviral vector in these cell lines are located at the different genetic loci. We are isolating their genomic DNAs for Southern blots in order to verify this view.

MATERIALS, METHODS, AND PROCEDURES

Cell Culture. The parental breast cancer cell line CAL51 and the chromosome-17 suppressed cell sublines CAL/17-1, CAL/17-3, and CAL/17-5 (37) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin G sodium, and 100 ug/mL streptomycin sulfate. Six hundred ug/mL of G418 and 10 ug/mL insulin were added in culture of the chromosome 17-containing cells. The insulin-independent revertant cell sublines are cultured in the absence of the insulin. Bosc23 cells (52) and Bing (53) cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin G sodium, and 100 ug/mL streptomycin sulfate. Ten% of newborn calf serum was used for culture of GP+envAM12 cells (54). Eight mM of L-histidinol dihydrochloride ($hisD^R$) was used for cells containing retrovirus vectors. All media, serum, and antibiotics were from Gibco BRL with exceptions where indicated. *Escherichia coli* strain DH5α cells (GIBCO, BRL) containing plasmid pLM2 (38) and strain Supercompetent cells (Catalogue no.

230140; Stratagene) containing a cDNA library were cultured in Luria-Bertani medium with 100 ug of ampicillin per ml.

Techniques of the Molecular Cloning. Genomic DNA and total RNA were isolated by standard methods (55). Poly(A)$^+$-RNA was extracted from total RNA using the FastTrack 2.0 mRNA Isolation Kit according to manufacturer's instructions (Catalog nos. K1593-02, K1593-03; Invitrogen). The cDNA library was synthesized from poly(A)$^+$ RNA using Universal RiboClone cDNA Synthesis System (Catalog no. C4360; Promega) according to the manufacturer's instruction. Briefly, first strand synthesis was driven by Avian Myeloblstosis Virus (AMV) reverse transcriptase and random hexameric primers, followed directly by second strand replacement synthesis using Rnase H and DNA polymerase I. After treatment with T4 DNA polymerase to flush the ends, the double-stranded cDNA molecules were prepared for cloning by size fractionation and the additino of *Eco*RI adaptors. The resulting cDNA samples were cloned into pLM2 plasmid vectors. To generate high transformation efficiency, the double stranded cDNA were phosphorylated and pLM2 vectors were dephosphorylated before ligation. Plasmid DNA was isolated using alkaline lysis methods (56) or using QIAGEN Plasmid Purification Kit (catalogue No. 12262) according to the manufacturer's instruction.

Packaging of Retrovirus Particles. Virus particles containing retroviral vectors were packaged from pLM2 plasmids, using the retrovirus-packaging cell lines Bosc-23 (ecotropic) and GP+envAM12 (amphotropic) and BING (amphotropic) by methods essentially as described (52-54). Briefly, 50 ug of plasmid DNA or pLM2-carried cDNA library were used to transfect approximately 10^7 Bosc-23 or BING cells using Cell-Porator Electroporation System I (catalogue No. 71600-019; GIBCO BRL) according to the manufacturer's instruction. To determine the transfection efficiency, 50-ug plasmid vectors with a green fluorescence gene was transfected into the same cells under the same conditions. 10 ml of supernatant from transfected Bosc-23 cells were also used to transduce aliquots of 1 x 10^6 GP+envAM12 cells in the presence of 6 ug Polybrene per ml (54) for production of the amphotropic virus particles. The pLM2-virus vector-containing cells are selected for with with L-histidinol. The amphotropic virus particles from packaged from the Bing cells were used to transduce the breast cancer cell lines.

Transduction. 15 T75-flasks of the chromosome 17-mediated suppressed cells (CAL/17-1) were cultured up to 50% confluence (approximately 10^7 cells/flask) and transduced with amphotropic virus particles in the transduction medium. The aliquots of 10 ml transduction medium consist of 5 ml culture medium and 5 ml supernatant from the transduced BING cells. 4-ug polybrene were added in each ml medium to enhance the attachment of the virus particles to the cell surface. The fresh transduction medium was used to replace the old one every 4 hours per day for three days. The transduced cells were selected for with both G418 and HisD for 10 days.

Soft agar selection. Approximately 3 x 10^6 transduced cells were cultured in 0.33% soft agarose based on 0.9% bottom agarose in the concentration of 50,000 per 60-mm dish. After 4-week culture, all the cells from the top agarose were combined for the second round of the soft agar selection to enrich the true positive anchorage-independent

cells. After the second round selection, more than 100 cell sublines are cultured, at this moment, in dishes for cell expansion and further analysis.

Selection for insulin-independent cell sublines. Approximately 5 x 10^6 transduced cells were cultured in the medium without insulin for two months to select for insulin-independent cell sublines. The CAL51 and CAL/17-1 cells were used as the positive and negative controls, respectively. At this moment, 7 insulin-independent cell lines are in culture for further analysis.

(7) KEY RESEARCH ACCOMPLISHMENT

This research has generated the following key research accomplishment.

a. The pLM2 plasmid-carried cDNA library from the chromosome 17-mediated suppressed breast cancer cell line CAL/17-1 is now available for many purposes.

b. Both the pLM2 plasmid vectors and the pLM2-carried cDNA library have been introduced into BOSC23, GP+am12, and BING cell lines for packaging the virus particles.

c. The suppressed cell line CAL/17-1 and the control cell line CAL51 have been transduced with the packaged virus particles.

d. More than 100 individual anchorage-independent cells have been selected from the soft agar assays. These cells are in plastic culture for cell expansion and further analysis.

e. In addition to the accomplishment of above proposed work, we have also selected for insulin-independent cells from the transduced CAL/17-1 cells. At this moment, 7 insulin-independent cell sublines display significantly different growth rates and are in expansion for further analysis.

(8) REPORTABLE OUTCOMES

The following results are reportable in near future.

a. The development of breast cancer cell sublines such as the anchorage-independent cell sublines and the insulin-independent cell sublines.

b. The preparation of the abstract and manuscript on the development of these cell sublines is in progress. This will be submitted to DOD U.S. Army Medical Research and Materiel Command Breast Cancer Research Program "Era of Hope Meeting" in June 8-12, 2000.

(9) CONCLUSIONS

a. Our preliminary results demonstrate that we have successfully applied our retrovirus-reversion strategy developed in malignant melanoma system to the chromosome 17-mediated suppressed breast cancer cell line.

b. The resulting anchorage-independent breast cancer cell sublines will be unique and very valuable for study of breast cancer progression including the identification of genes involved in this reversion.

c. The successful generation of the insulin-independent breast cancer cell sublines not only demonstrates another application of our retrovirus-reversion strategy but also provides unique and valuable cell resource for study of the growth factor dependent and independent biology.

d. Our results on the successful generation of the anchorage-independent and the insulin-independent cell sublines strongly suggest that the retrovirus-reversion strategy can be applied to many areas. For example, we can select for the hormone-independent cell sublines from a hormone-independent cell line, the tumorigenic cell sublines from a non-tumorigenic cell line, and the metastatic cancer cell sublines from a non-metastatic cell line.

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